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Metabolomics meets systems immunology

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Abstract

Metabolic processes play a critical role in immune regulation. Metabolomics is the systematic analysis of small molecules (metabolites) in organisms or biological samples, providing an opportunity to comprehensively study interactions between metabolism and immunity in physiology and disease. Integrating metabolomics into systems immunology allows the exploration of the interactions of multilayered features in the biological system and the molecular regulatory mechanism of these features. Here, we provide an overview on recent technological developments of metabolomic applications in immunological research. To begin, two widely used metabolomics approaches are compared: targeted and untargeted metabolomics. Then, we provide a comprehensive overview of the analysis workflow and the computational tools available, including sample preparation, raw spectra data preprocessing, data processing, statistical analysis, and interpretation. Third, we describe how to integrate metabolomics with other omics approaches in immunological studies using available tools. Finally, we discuss new developments in metabolomics and its prospects for immunology research. This review provides guidance to researchers using metabolomics and multiomics in immunity research, thus facilitating the application of systems immunology to disease research.

Keywords infection; metabolomics; multiomics; statistical analysis; systems immunology

Subject Categories Computational Biology; Immunology; Metabolism

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Introduction

Infections are significant threats to global public health (Duff *et al*, 2021). Lower respiratory infections, human immunodeficiency virus (HIV) infection, malaria, and tuberculosis are associated with higher mortality rates (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death; Liu *et al*, 2022a). The rapidly spreading coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a potentially fatal respiratory infection (COVID-19 National Preparedness Collaborators, 2022; Zhang *et al*, 2022). As of June 16, 2022, the World Health Organization (WHO) reported over 530 million confirmed cases and 6 million deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Therefore, there is an urgent need to better understand the molecular pathophysiology of infectious diseases, in order to inform treatment and management strategies, including the development of new therapies. Studies have shown that immunity has played a crucial role in protecting against infectious diseases such as COVID-19 and HIV infection (Kardava *et al*, 2018; Herzig *et al*, 2019; Combes *et al*, 2021; Schultze & Aschenbrenner, 2021; Witkowski *et al*, 2021). Researchers have observed various physiological and immunological changes, including the complex and dynamic nature of immune responses, in COVID-19 patients (Arunachalam *et al*, 2020; Woodruff *et al*, 2020; Krause *et al*, 2021) and HIV (Ringeard *et al*, 2019; Campion *et al*, 2021; Goh *et al*, 2022). Systems immunology, which complements traditional approaches, has become a valuable tool for understanding the complex immune responses to infectious diseases (Dunning *et al*, 2019; Warsinske *et al*, 2019; di Iulio *et al*, 2021; Koeken *et al*, 2021; Potapov *et al*, 2022).

Systems immunology explores the interactions between cytokines, chemokines, cells, and molecular networks, as well as the dynamic changes in the immune system using mathematical and computational methodologies (Villani *et al*, 2018). One approach in systems immunology is to develop mathematical models and improve them through inference to understand the functionality of the immune system (Eftimie *et al*, 2016). In recent years, various modeling methods have been developed for immune cells, including ordinary differential equation models (Kim *et al*, 2009), partial differential equation models (Cemerski *et al*, 2007), the particle-based stochastic models (Boianelli *et al*, 2015), the agent-based models (Tang & Hunt, 2010), and the Boolean models (Keef *et al*, 2017). Some computational tools have also been developed for modeling a system, including GINsim (Chaouiya *et al*, 2012), Boolnet (Mussel *et al*, 2010), Cell Collective (Helikar *et al*, 2013), BioNetGen (Harris *et al*, 2016), and DSAIRM (Handel, 2020). Handel *et al* (2020) summarized in detail the utility of mechanistic simulation models in the field of immunology. Cappuccio *et al* (2016) reviewed the recent advancements in

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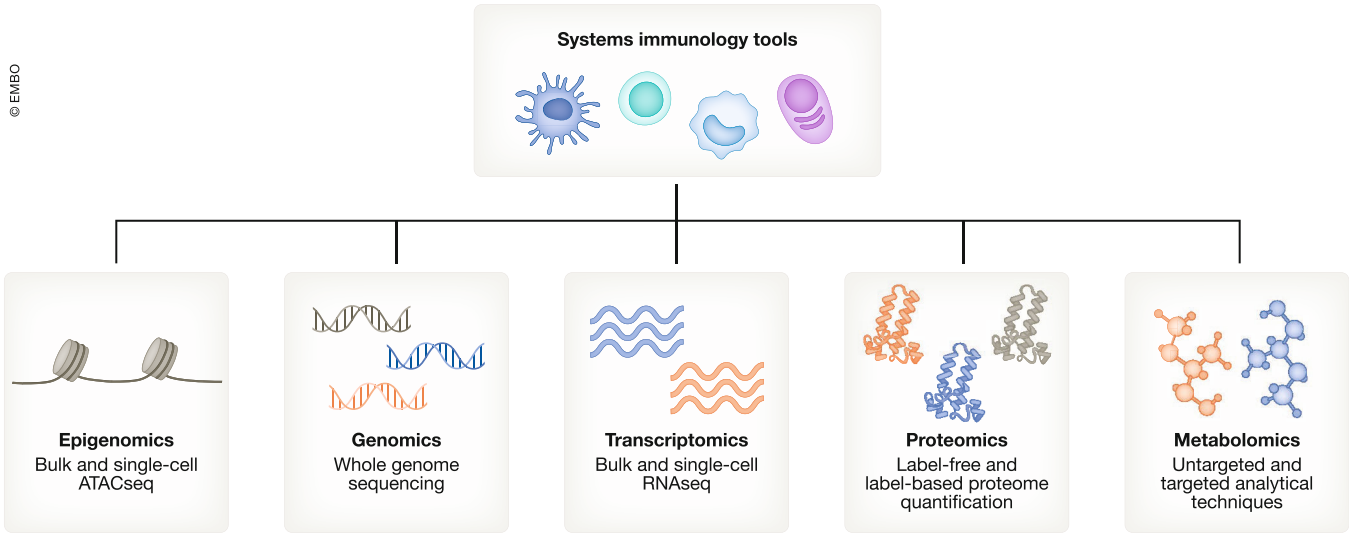

Figure 1. Overview of omics-based technologies in systems immunology.

This figure shows different omics-based technologies used in systems immunology, including but not limited to epigenomics (bulk and single-cell ATAC-Seq), genomics (whole-genome sequencing or genome-wide genotyping), transcriptomics (bulk and single-cell RNA-Seq), proteomics (label-free and label-based proteome quantification), and metabolomics (untargeted and targeted mass spectrometry for measuring metabolite levels). These technologies offer a comprehensive understanding of the immune system and support research in systems immunology.

multiscale models, which can be used to describe therapeutic treatments of complex immune diseases.

In the past decade, the rapid development of high-throughput omics technologies has enabled researchers to obtain a large amount of data from very limited samples, such as blood, secretions, or tissue biopsies, promoting the field of systems immunology and aiding in the exploration of the immune system (Davis *et al*, 2017; Tomic *et al*, 2021). Several omics-based technologies, including but not limited to epigenomics (assay for transposase-accessible chromatin using sequencing (ATAC-Seq) for identifying chromatin accessibility across the genome), genomics (whole-genome sequencing or genome-wide genotyping), transcriptomics (RNA-sequencing (RNA-Seq) for measuring levels of gene expression and transcripts), proteomics (proteomics involves the broad applications of technologies for the identification and quantification of proteins, protein post-translational modifications, protein–protein interactions, and subcellular protein localization), and metabolomics (untargeted and targeted mass spectrometry for measuring metabolite levels), as well as single-cell omics analysis (measure various molecules at the single-cell level), have been used in systems immunology (as shown in Fig 1). Unlike other omics-based tools, metabolomics is terminally downstream of the products of the genome and provides the closest connection to the phenotype of cells and organisms (Clish, 2015; Wishart, 2016). Metabolites and metabolic enzymes play an important role in the regulation of immune cells (Artyomov & Van den Bossche, 2020). Thus, metabolomics can help to understand the mechanisms of immune cell metabolism and disease progression. Furman *et al* (2014) used systems analysis to identify the lipid metabolism and endocrine components that inhibit immune system function. They also used systems analysis and metabolomic analysis to find abnormal nucleotide metabolism in their longitudinal cohort (Furman *et al*, 2017) and showed how to use systems methods to establish a network to gain a better understanding of a common human disease with unclear pathogenesis (reviewed in Davis *et al*, 2017). Some groups have linked immunological with metabolomic data to identify major immunological and other changes that have occurred (Ghaemi *et al*, 2019; Apps *et al*, 2020), which plays a critical role in further studying pregnancy (reviewed in Davis, 2020).

Metabolomics is a technique used to analyze metabolites, which are small molecules produced by an organism, in biological samples. It can be used to study the response of an organism to an internal (genetic) or external (environmental) stimulus (Johnson *et al*, 2016; Azad & Shulaev, 2019). Metabolomics has become a powerful analytical tool in systems biology and is gaining popularity in the field of systems immunology. For example, Abdrabou *et al* (2021) used global metabolomics to identify the role of steroids as a key class of metabolites that have an impact on the immune response of the *P. falciparum* infection. Diray-Arce *et al* (2022) employed mass spectrometry-based metabolomics of blood plasma and found that Bacille Calmette-Guerin induced changes to the plasma lipidome and lysophosphatidylcholines are relevant to vaccine immunogenicity. Lee *et al* (2022) showed a strong interaction between plasma metabolites and metabolic reprogramming networks in the immune response to COVID-19. In brief, metabolomics enables researchers to identify a key set of biomarkers by analyzing changing metabolic patterns (Weiner *et al*, 2018; Shen *et al*, 2020; Thomas *et al*, 2020) and understand the interactions between metabolic pathways and immune responses in infectious diseases (Li *et al*, 2018; Chan *et al*, 2019; Saez-Cirion & Sereti, 2021; Xiao *et al*, 2021). The rapid development of MS and/or NMR-based methods has enabled scientists to analyze thousands of metabolites and better understand the regulation of metabolic networks in infection and other diseases (Ulas *et al*, 2017; Weiner *et al*, 2018; Correia *et al*, 2022; Yan *et al*, 2022). However, processing mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) data obtained from biological

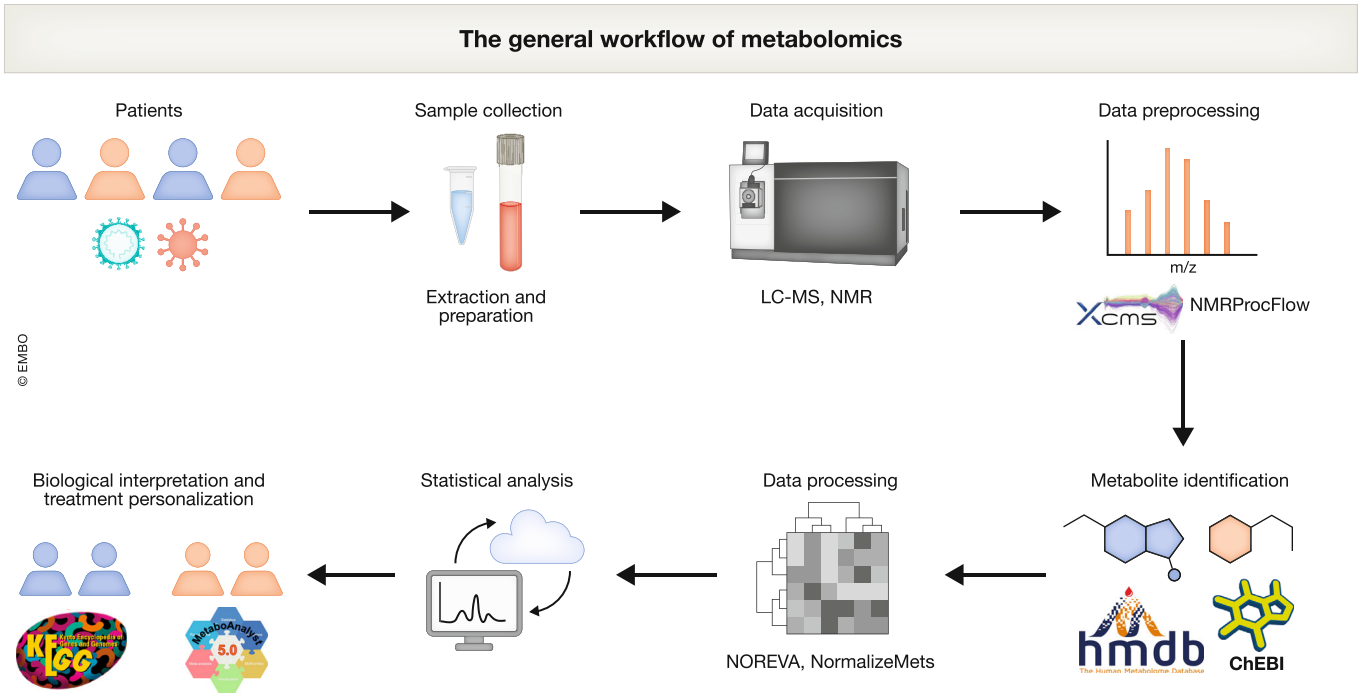

Figure 2. Metabolomics workflow in immunological studies.

The general workflow includes sample collection (sample preparation and extraction), data acquisition (detection by MS or NMR), data preprocessing (the curation of raw MS signals acquired from various acquisition platforms), metabolite identification (metabolite identification and verification using metabolome datasets), data processing (quality control samples correction, normalization, and missing value imputation), statistical analysis (the use of feature selection techniques to identify biological biomarkers), and biological interpretation (the interpretations of metabolic pathways and multilayer networks to understand disease mechanisms).

samples, as well as dealing with many background signals and noise, can be very challenging (Han *et al*, 2017; Wanichthanarak *et al*, 2019). So far, a large number of computational software, statistical algorithms, and databases, which are crucial for the qualitative and quantitative characterization of metabolites, have been developed, which further promotes the use of metabolomics in immunology research and infection (Tautenhahn *et al*, 2012; Wishart *et al*, 2018; Everett *et al*, 2019).

In this review, we provide an overview of metabolomics studies that investigate immunity focusing on recent technological advances in mass spectrometry. The overall metabolomics workflow (as shown in Fig 2) encompasses the following steps: sample collection (the extraction and preparation of samples), data acquisition (detection by MS or NMR), data preprocessing (the curation of raw MS signals acquired from differential acquisition platforms), metabolite identification (the identification and validation of metabolites from metabolome databases), data processing (quality control samples correction, normalization, and missing value imputation), statistical analysis (the application of feature selection approaches to find biological biomarkers), and biological interpretation (the interpretations of metabolic pathways and multilayer networks to understand disease mechanisms; Xia & Wishart, 2011; Tugizimana *et al*, 2016; Pulendran & Davis, 2020; Wozniak *et al*, 2020; Fu *et al*, 2022). Therefore, there are three components that follow. First, this review compares targeted and untargeted metabolomics and reviews recent advances in metabolomics workflows, including sample preparation, raw spectra data preprocessing, metabolomics data processing, statistical analysis, and interpretation. Second, we introduce various computational tools for multiomics data integration and show how recent studies have applied metabolomics in combination with other omics approaches to immunological research. Finally, we discuss new developments in metabolomics and their potential for further research in immunology.

Targeted and untargeted metabolomics for immunology research

In metabolomics research, metabolites are analyzed by extracting compounds from plasma, urine, tissues, etc., and then identifying and quantifying compounds using different analytical platforms such as liquid chromatography–mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS), and NMR. There are two main types of metabolomics approaches: targeted and untargeted (Jacob *et al*, 2019; Gonzalez-Riano *et al*, 2020). Targeted metabolomics focuses on identifying specific know metabolites, while untargeted metabolomics aims to examine a wide range of known and unknown metabolites. Fig 3A illustrates the difference between these two approaches.

Targeted metabolomics

The targeted metabolomics approach focuses on the analysis of specific, predetermined metabolites, as opposed to a comprehensive

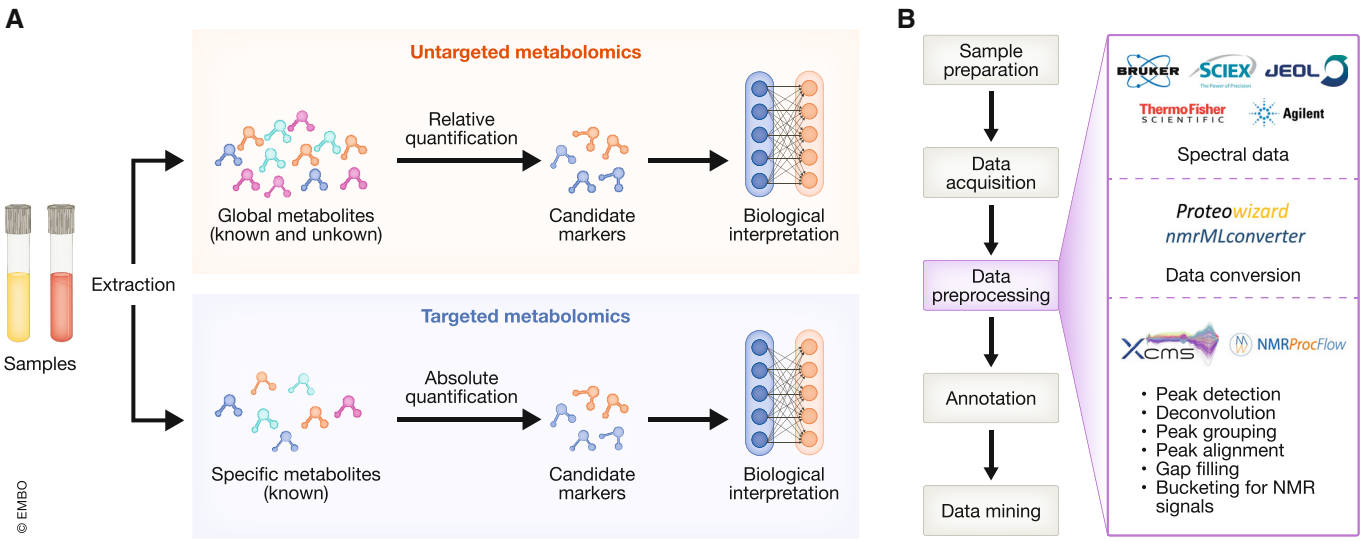

Figure 3. Mainstream metabolomics techniques in immunology.

(A) An overview of two different metabolomics approaches: untargeted and targeted metabolomics. The untargeted metabolomics aims to study a wide range of known and unknown metabolites, whereas targeted metabolomics focuses on identifying specific known metabolites. (B) Data preprocessing Workflow. For NMR data, common preprocessing steps include peak detection, phase correction, baseline correction, peak alignment, and bucketing. For MS data, the general preprocessing workflow encompasses peak detection, deconvolution, peak grouping, peak alignment, and gap filling.

analysis of all known metabolites (Johnson *et al*, 2016; Gross *et al*, 2018). This approach primarily uses internal standards for qualitative and quantitative analysis of compounds. In addition, the use of isotopic internal standards can enhance the sensitivity and accuracy of metabolite detection. The preferred analytical platform of targeted metabolomics is multiple reaction monitoring (MRM) with LC–MS, which is useful to quantify known metabolites. This approach has the significant advantages of high specificity, sensitivity, and quantitative accuracy for in-depth exploration and analysis of subsequent metabolic molecular markers and the specific metabolic pathways, which play a significant role in disease research and diagnosis (Griffiths *et al*, 2010; Cao *et al*, 2020; Karnovsky & Li, 2020).

Targeted metabolomics has been used to screen and verify biological markers of immunity and infectious diseases (Neugebauer *et al*, 2016; Kuhn *et al*, 2018; Cho *et al*, 2020; Lopez-Hernandez *et al*, 2021). Ansone *et al* (2021) used targeted metabolome analysis with LC–MS and found that tryptophan and arginine metabolism may be involved in the immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Magdalena *et al* (2022) used targeted metabolomics analysis to identify small metabolites as biomarkers of childhood *Mycobacterium tuberculosis* infection. Ma *et al* (2022) employed targeted metabolomics to validate the metabolites in patients with sepsis infection and understand the function of host Cytochrome P450 Family 1 Subfamily A Member 1 (CYP1A1). Targeted metabolomics has also contributed to the study of metabolic mechanisms (Babu *et al*, 2019; Zou *et al*, 2020; Haimerl *et al*, 2021). Marín-Corral *et al* (2021) used targeted metabolomics to evaluate metabolic changes associated with disease severity in COVID-19 and found the pathways related to ceramide, tryptophan, and nicotinamide adenine dinucleotide (NAD) production, as well as an exacerbated pro-inflammatory response, may be relevant to disease severity. Peltenburg *et al* (2018) used targeted metabolomics on plasma samples and identified significant immune-metabolic changes in HIV-infected patients. In the study of Noto *et al* (2022), targeted metabolomics revealed that altered bile acid metabolism may contribute to *Helicobacter pylori*-induced inflammation-driven gastric cancer.

Immunometabolism is a complex and dynamic process (Mathis & Shoelson, 2011; O'Neill *et al*, 2016). Traditional metabolomics methods are limited in their ability to track changes in specific metabolic pathways, but stable isotope labeling metabolomics can overcome this limitation. This technique involves labeling specific compounds with stable isotopes (as discussed in Jang *et al*, 2018). By analyzing the stable isotope labeling of downstream metabolites, researchers can deduce the distribution of intracellular metabolic pathways and, by analyzing organisms in different states, determine the activity of specific metabolic pathways. Stable isotope labeling metabolomics has been widely used in the research of metabolic-related diseases such as cancer and immune-related diseases, providing a strong scientific foundation for understanding the pathogenesis of these diseases and identifying potential drug targets (Davidson *et al*, 2016; Faubert *et al*, 2017; Ma *et al*, 2019; Yuan *et al*, 2019; Sheldon *et al*, 2021).

Untargeted metabolomics

The untargeted metabolomics approach involves the unbiased detection of small molecule metabolites (including unknown chemicals) using LC–MS, GC–MS, and NMR platforms (Schrimpe-Rutledge *et al*, 2016). Differential metabolites are then analyzed using bioinformatics tools, and pathway analysis is performed to elucidate the physiological mechanisms of the changes that have occurred. Untargeted metabolomics approaches involve the comprehensive analysis of global metabolites and often provide more information than

targeted metabolomics, enabling the discovery of new biomarkers or the identification of differential metabolites (Sevin *et al*, 2015). This approach is currently widely used in the discovery of biomarkers, disease diagnosis, and mechanism research, and it provides new insights into the complex study of disease mechanisms (Haimerl *et al*, 2021; Krishnan *et al*, 2021; Roberts *et al*, 2021; Choueiry *et al*, 2022).

Untargeted metabolomics can reveal important biological associations in immunology research and identify novel metabolites/biomarkers specific to a disease (Ding *et al*, 2021b; Alboniga *et al*, 2022; Aximujiang *et al*, 2022; Chen *et al*, 2022; Tian *et al*, 2022). For example, Zhu *et al* (2022) used high-resolution untargeted metabolomics analysis to study influenza A virus subtype H1N1 (H1N1) infected patients and found an association between abnormal arginine metabolism produced by the virus and the immunity of the respiratory mucosa. She *et al* (2022) used untargeted metabolomics analysis to discover metabolic hub genes that may be biomarkers for precise therapy in sepsis patients. In their research, Ding *et al* (2021b) used untargeted metabolomics analysis to identify glycerophospholipid (GPL) metabolism and glutamine and glutamate metabolism as potential key targets in HIV infection.

Both metabolomics approaches have their advantages and disadvantages. Untargeted metabolomics provides a comprehensive and systematic response to the metabolome, while targeted metabolomics is more focused on the quantitation of specific metabolites. In practical applications, both approaches are often used in combination to complement each other (Asim *et al*, 2020; Thomas *et al*, 2020; Jia *et al*, 2022; Mikaeloff *et al*, 2022; Yelamanchi *et al*, 2022). For example, in the study of Xiao *et al* (2021), the combination of untargeted and targeted metabolomics revealed that disturbed metabolic pathways associated with hyperinflammation in severe COVID-19 could be targeted with metabolic interventions as a possible approach to inhibit SARS-CoV-2-induced cytokine release syndrome. Tarancon-Diez *et al* (2019) used both targeted and untargeted approaches to show that the specific metabolomic profile could be a potential biomarker and therapeutic target in HIV infection. Vrieling *et al* (2020) combined targeted NMR and untargeted LC–MS metabolomics to explore the effect of *Mycobacterium tuberculosis* infection on primary human macrophage metabolism.

Sample preparation

The extraction of metabolites and sample preparation in metabolomics research can affect the analyzed metabolic characteristics and the biological interpretation of the metabolomic data (Tulipani *et al*, 2013; Naz *et al*, 2014). Metabolites have a rapid turnover rate, and changes in the sample collection period (such as day and night), as well as in temperature, light, and mechanical pressure (such as centrifugation and vortex) after collection, can impact the stability of metabolites (Zhang *et al*, 2013; Lu *et al*, 2017). Different solvents and different extraction protocols can produce different results for metabolite identification (Le Belle *et al*, 2002; Wu *et al*, 2008; Chen *et al*, 2013). Ideally, the metabolomic analysis should aim to extract as many metabolite classes as possible, using both polar (such as methanol or ethanol) and nonpolar (such as ethyl acetate, hexane, and chloroform) solvents. However, no single extraction method can extract all metabolites equally well (Hyotylainen, 2009; Naz *et al*, 2014). Naz *et al* (2014) reviewed many different previously developed sample preparation protocols and metabolomic measurements of tissue samples in detail.

Reverse phase (RP) and hydrophilic interaction chromatography (HILIC) are the most commonly used chromatographic methods for LC–MS-based metabolomics (Le *et al*, 2020). HILIC has several advantages over RP for analyzing polar compounds, including higher retention of polar metabolites and enhanced mass spectrum sensitivity (Buszewski & Noga, 2012). The human metabolome includes lipids, carbohydrates, and metabolic intermediates (such as organic acids, amino acids, and acylcarnitines; Wishart, 2019). RP coupled with mass spectrometry has been used to identify a wide range of nonpolar compounds, but it is not as effective at analyzing carbohydrates, organic acids, amino acids, and nucleotides. These compounds can be well preserved and separated using HILIC. Since RP or HILIC-based methods may miss some key metabolites (Wikoff *et al*, 2007; Miller *et al*, 2015; Coene *et al*, 2018), the results of these two methods are often combined using full scan MS to capture and detect all compounds (Want *et al*, 2010; Granafei *et al*, 2016; Gao *et al*, 2018). Jang *et al* (2018) summarized in detail the different isotope tracers and their utility for sample preparation in stable isotope labeling metabolomics.

Data preprocessing and processing methods applied on metabolomics data

Both targeted and untargeted metabolomics rely on analytical platforms such as MS and NMR, and these technologies have played a significant role in immunological research. However, these metabolomic analyses often face challenges such as signal drift, experimental technical errors, missing values, and biological variability, which can be very challenging for researchers to address (Han *et al*, 2017; Wanichthanarak *et al*, 2019; Andres *et al*, 2020). Currently, a variety of powerful tools and computational algorithms have been developed for data processing, which can help to reduce these challenges and are widely used in immunology research. Understanding these tools can help researchers select the most appropriate tools, make better use of them, and apply them in scientific research.

Spectral data preprocessing

Different analytical platforms are used for separating and detecting metabolite analytes, which produces complex raw spectral data. To prepare this data for annotation and statistical analysis (as shown in Fig 3B), computational tools are needed for spectral data preprocessing. Spectral data are primarily generated by two analytical platforms: NMR and MS. The preprocessing of spectral data for these two platforms is different. The conversion of spectral data, generated by various analytical platforms developed by different vendors, to open-source format files can be accomplished using tools such as nmrML Converter (http://nmrml.org/) for NMR data (Schober *et al*, 2018). The nmrML Converter supports formats from Bruker, JEOL, and Agilent/Varian and can capture raw NMR data, the spectral data acquisition parameter, and metadata, and offers easy-to-use web-based tools (Schober *et al*, 2018). For MS data, common conversion tools include Proteowizard (Chambers *et al*, 2012), MassLynx (Wang *et al*, 2012), and CompassXport (Kasalica *et al*, 2021). Proteowizard converts original MS data into mzXML format,

which allows for the distribution of vendor-supplied libraries from AB SCIEX, Agilent, Bruker, Thermo Fisher Scientific, and Waters (Chambers *et al*, 2012). MassLynx can convert Waters raw files to mzXML format (Wang *et al*, 2012). CompassXport converts raw files from Bruker and some Agilent to the universal mzXML format (Kasalica *et al*, 2021).

After converting spectra data, different procedures will be used to preprocess NMR and MS data. For NMR data, common preprocessing steps include peak detection, phase correction, baseline correction, peak alignment, and bucketing. Tools commonly used for NMR data include NMRProcFlow (Jacob *et al*, 2017), BATMAN (Hao *et al*, 2014), and MNova (Claridge, 2009). NMRProcFlow covers all spectral processing steps, including baseline correction, chemical shift calibration, and alignment. (Jacob *et al*, 2017). BATMAN deconvolves and quantifies metabolites in complex mixtures using a Bayesian model of 1D NMR spectra (Hao *et al*, 2014), and Mnova, which is primarily aimed at 1D/2D spectra of small/medium-sized molecules, includes all spectra preprocessing steps such as phase correction, baseline correction, peak alignment, and bucketing (Claridge, 2009). For MS data, the general preprocessing workflow consists of the following steps such as peak detection, deconvolution, peak grouping, peak alignment, and gap filling.

There are several tools that are widely used for MS data preprocessing, including XCMS (Smith *et al*, 2006), Progenesis (Zhang *et al*, 2016), OpenMS (Rost *et al*, 2016), MAVEN (Clasquin *et al*, 2012), and MZmine (Pluskal *et al*, 2010). To preprocess MS data for metabolite profiling, XCMS employs nonlinear retention time alignment, matched filtration, peak detection, and peak matching (Smith *et al*, 2006). Progenesis is used for mass spectrometry data preprocessing, including baseline correction, smoothing, deconvolution, and peak alignment (Zhang *et al*, 2016). In OpenMS software, the preprocessing steps include isotope deconvolution, chromatographic peak picking, retention time alignment (RT), and feature linking across multiple runs (Rost *et al*, 2016). MAVEN is an interactive MS data processing tool that automatically discovers and investigates peak intensities for isotope-labeled metabolites (Clasquin *et al*, 2012). MZmine has been employed in both targeted and untargeted metabolomic analyses, including noise reduction through chromatographic filtering, raw data range cropping, and scan removal based on width (Pluskal *et al*, 2010). Thermo Scientific TraceFinder software is popular for Thermo instruments, offering comprehensive qualitative workflows for LC and GC–MS data that include smart calibration curves, library search capability, automatic retention time and ion ratio adjustment, and extensive flagging options.

Data processing

The peak intensity table will be generated after preprocessing the raw spectra data, which includes procedures such as peak identification, peak alignment, calibration, and others. There are many challenges in this process, including missing data, uninformative features, data skewness, and systematic bias due to instrumentation or sampling issues. Therefore, data processing is necessary to improve the quality of the data (Craig *et al*, 2006; Zelena *et al*, 2009; Dunn *et al*, 2011). In general, data processing involves five key steps: (i) data filtering, which removes uninformative or low-quality features from metabolomic data; (ii), missing value imputation, which calculates and replaces missing values to ensure data integrity and enable statistical analysis; (iii) quality controls correction (QCS), which corrects signal drifts and batch variations to ensure data quality and stability; (iv), data transformation, which reduces heteroscedasticity and corrects skewed distribution, making the data distribution symmetrical, and satisfying the assumptions of normalization methods; and (v) normalization, which reduces unwanted systematic bias and makes data comparable among samples or metabolites, improving the reliability of the statistical analysis.

At present, there are several tools available for processing metabolomic data. Some of these tools cover the entire metabolomics workflows, including the data processing step, such as IP4M (Liang *et al*, 2020), KIMBLE (Verhoeven *et al*, 2018), MetaboAnalyst (Xia & Wishart, 2011), MetaDB (Franceschi *et al*, 2014), Metandem (Hao *et al*, 2019), MetFlow (Shen & Zhu, 2019), Workflow4Metabolomics (Giacomoni *et al*, 2015), WebSpecmine (Cardoso *et al*, 2019), and XCMS (Forsberg *et al*, 2018). These tools have also been used in immunology research. For example, Zhang *et al* (2021) employed MetaboAnalyst to process metabolomics data and revealed that metabolic reprogramming affects an anti-inflammatory phenotype with trained immunity. Montenegro-Burke *et al* (2021) used XCMS in the data processing step and found distinct anti-inflammatory metabolites from T cell-induced colitis. Moreau *et al* (2020) applied Workflow4Metabolomics tools in the data processing and uncovered inflammation-related mitochondrial dysfunction as a potential mechanism underlying liver failure. Wei *et al* (2019) used Metandem to process metabolomics data and identified metabolites biomarkers as well as inflammation-related biological processes for inflammation-induced Lower urinary tract symptoms. Knoll *et al* (2021a) used MetFlow in the data processing and elucidating the antimycobacterial mechanism of action of ciprofloxacin. Some tools are specialized in a specific procedure of data processing, such as batchCorr (Brunius *et al*, 2016) and MetaboQC (Calderon-Santiago *et al*, 2017). There are also several tools that provide not only data processing but also performance evaluation of processing workflows, including MetaboGroup S (Wang *et al*, 2018), metaX (Wen *et al*, 2017), MSPrep (Hughes *et al*, 2014), NOREVA (Fu *et al*, 2022), NormalizeMets (De Livera *et al*, 2018), NormalyzerDE (Willforss *et al*, 2019), and pseudoQC (Wang & Yang, 2019). For example, Hartvigsson *et al* (2021) performed untargeted metabolomics and then used batchCorr tool to process the data, discovering the relationships between maternal and infant metabolic profiling with immune maturation and allergy development. Lee *et al* (2019) applied the NOREVA to process metabolomics and found metabolic biomarkers that could accurately predict adverse pregnancy outcomes in patients with systemic lupus erythematosus. Zeng *et al* (2017) performed metaX to process metabolomics data and uncovered the function of glycerophospholipid metabolism in psoriasis. In the study by Bowerman *et al* (2020), the NormalizeMets tool was used to process untargeted metabolomics data, helping to identify changes in the metabolites in patients with pulmonary disease.

Statistical analysis and interpretation tools for metabolomics in immunology research

Following the step of data processing, statistical analysis and data interpretation will be applied to identify metabolic markers and

assess the relationships between metabolite features and immunological or clinical phenotypes. This step mainly includes statistical analysis, pathway, and network analysis. Many tools have been developed to aid in statistical analysis for the discovery of metabolic biomarkers and data interpretation. The following tools are commonly used to analyze metabolomics data and have a variety of statistical methods.

MetaboAnalyst can be used to analyze and interpret metabolomics data in depth (Chong *et al*, 2018). It includes methods of univariate analysis such as fold change analysis (FC), analysis of variance (ANOVA), and Student's *t*-tests. The advanced statistical analysis approaches include significance analysis of metabolites (SAM) and empirical Bayesian analysis of metabolites (EBAM). The chemometrics analysis methods such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (orthoPLS-DA), and sparse partial least squares discriminant analysis (sPLS-DA) are also widely used (Rio *et al*, 2009; Miolo *et al*, 2016; He *et al*, 2018). Clustering analysis approaches including hierarchical clustering, K-means, and self-organizing map (SOM), as well as classification and feature selection such as random forest (RF) and support vector machine (SVM), are commonly applied to metabolomics studies (Bartel *et al*, 2013; Li *et al*, 2016; Broughton-Neiswanger *et al*, 2020). MetaboAnalyst also offers biological interpretation tools such as pathway analysis, enrichment analysis, and network analysis. It has been widely applied in immunological research, for example, Silva *et al* (2019) used it to analyze plasma metabolites that may distinguish HIV-TB patients with and without tuberculosis-related immune reconstitution inflammatory syndrome (TB-IRIS).

The KIMBLE platform, which is built on open-source data mining and workflow technology, can be used to analyze targeted and untargeted NMR data (Verhoeven *et al*, 2018). It has implemented PCA, hierarchical cluster analysis, and PLS-DA. The extensive node library provided by KIMBLE enables users with no programming experience to apply, modify, and expand workflow. At present, KIMBLE has been utilized to identify the metabolic profile (Deelen *et al*, 2019; Dekker *et al*, 2020) and to identify the key metabolic pathway of infection (Kokova *et al*, 2020).

MeltDB was primarily created for storing, managing, analyzing, and annotating MS-based metabolomics data (Neuweger *et al*, 2008). This tool has provided *t*-test, ANOVA, HCA (hierarchical cluster analysis), PCA, independent component analysis (ICA), and metabolite correlation analysis. In addition, MeltDB also includes pathway analysis and enrichment analysis. In the context of immunological studies, MeltDB aims to provide bioinformatics tools for interpreting and analyzing pathway information to advance research physiology and pathology (Martinez *et al*, 2013; Pathak & Dave, 2014; Ruwe *et al*, 2019).

PhenoMeNal is a tool that can process and analyze metabolomics data in the cloud (Peters *et al*, 2019). It not only provides statistical methods, including PLS-DA, RF, SVM, and PCA, but also performs pathway analysis. PhenoMeNal offers a number of standardized, automated, and publicly available analysis pipelines in the Galaxy, Jupyter, Luigi, and Pachyderm user interfaces. Galaxy Workflow is a platform for combining different methods and analyzing metabolomic data. In addition to PhenoMeNal, the following Workflow4-Metabolomics (W4M) also offers galaxy workflows. PhenoMeNal has been used in the discovery of biomarkers and mechanisms related to pathophysiological processes in immunology research (Abbiss *et al*, 2019; Rinschen *et al*, 2019).

W4M is based on the galaxy environment, which provides user-friendly functions for creating and running data analysis workflows such as pretreatment, statistical analysis, and annotation (Guitton *et al*, 2017). W4M has implemented several univariate test methods, including the *t*-test, Wilcoxon test, ANOVA, *Kruskal–Wallis* test, and *Pearson* or *Spearman* correlation test. It also provided multivariate analysis methods, such as PLS and its orthogonal variant (OPLS). The Galaxy module in W4M enables users to conduct both unsupervised (PCA) and supervised (PLS, OPLS, PLS-DA, and OPLS-DA) analyses. W4M has been applied to immunological research; for example, Moyne *et al* (2021) used it to identify bacterial metabolic profiles that are significantly associated with clinically relevant bacterial phenotypes and chronic infections in cystic fibrosis.

MetaX is a tool that provides an easy-to-use pipeline for analyzing MS metabolomics data (Wen *et al*, 2017). It offers several statistical methods, including univariate analysis methods (Wilcoxon test and *t*-tests), multivariate analysis (PCA, PLS-DA, OPLS-DA, and cluster analysis), biomarker analysis (RF and SVM), power analysis, correlation network analysis, functional analysis, and pathway analysis. MetaX helps users identify metabolic markers and pathways (Chen *et al*, 2018; Vasaikar *et al*, 2019) and is a crucial tool in the field of systems immunology.

MetFlow is a server that offers a comprehensive pipeline for processing data and discovering differential metabolites (Shen & Zhu, 2019). It provided common univariate and multivariate analyses. Unsupervised PCA reports the metabolome-wide difference between samples, whereas supervised PLS calculates variable influence on projection (VIP) values to evaluate individual metabolite contributions. In the study by Knoll *et al* (2021b), MetFlow was used to analyze metabolomics data and to elucidate the antimycobacterial mechanism of action of the decoquinate derivative RMB041 (Knoll *et al*, 2021b).

MMEASE is an online platform for metabolomic data meta-analysis that uses metabolite annotation, marker selection, and enrichment analysis (Yang *et al*, 2021). It offers several methods for sample separation, including hierarchical clustering, K-means clustering, PCA, and SOM. It also has several methods for identifying markers, such as FC, PLS-DA, OPLS-DA, *t*-test, chi-squared test, correlation-based method, entropy-based filters, linear model and Bayes, relief, random-forest-recursive feature elimination (RF-RFE), SAM, support vector machine-recursive feature elimination (SVM-RFE), and Wilcoxon rank-sum test. MMEASE, which is an important tool in immune research, provides users with metabolic markers and metabolic pathway analysis (Galezowska *et al*, 2021).

WebSpecmine is a web server that analyzes metabolomics data from various sources, including NMR, infrared, UV–visible, Raman, and LC/GC–MS (Cardoso *et al*, 2019). It not only offers various approaches but also allows for sharing of data and results. The server implements univariate statistical analysis methods such as *t*-tests, one-way ANOVA, Kruskal–Wallis, Kolmogorov–Smirnov, and FC. In addition, it offers supervised machine learning models, including linear discriminant analysis (LDA), PLS, SVM, neural networks (NN), and pathway analysis. WebSpecmine provides comprehensive metabolomic statistical analysis methods and may constitute a contribution to the systems immunology (Amer & Baidoo, 2021).

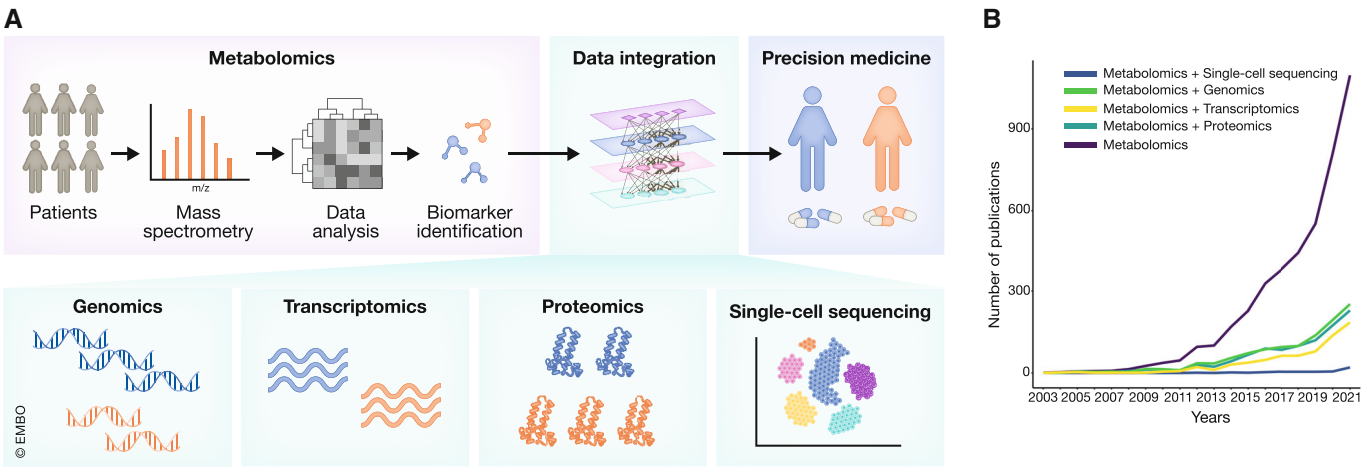

Figure 4. Integrative metabolomics in immunology research.

(A) Schematic depiction of the metabolomics integration workflow. By combining metabolomics with genomics, transcriptomics (single-cell), and proteomics, biological mechanisms can be explored from multiple perspectives. (B) Annual publications using the terms metabolomics, single-cell sequencing, genomics, transcriptomics, and proteomics in PubMed from 2003 to 2021. The purple, blue, green, yellow, and dark green lines represent only metabolomics and the combination of metabolomics with single-cell sequencing, genomics, transcriptomics, and proteomics, respectively.

IP4M is a platform for analyzing metabolomics data using MS (Liang *et al*, 2020). It provides a range of procedures for processing this data, as well as statistical analysis methods including univariant methods (Wilcoxon, ANOVA, and Kruskal–Wallis test), and multivariant methods (PCA, OPLS-DA, RF, SVM, Biosigner, and Boruta). It also includes machine learning methods, correlation analysis, cluster, and regression analysis, receiver operating characteristic curve (ROC) analysis, pathway and enrichment analysis, and the Generalized coRrelation analysis for Metabolome and Microbiome (GRaMM). IP4M could aid users in the discovery of new biomarkers for metabolic disorders (Zheng *et al*, 2021) and would be a useful tool in the field of systems immunology.

Integrative metabolomics in immunology research

Metabolomic analysis can identify different metabolic features and dynamic changes in response to various phenotypes, diseases, and environments (Olszewski *et al*, 2009; Gulati *et al*, 2015; Joice Cordy, 2020). Metabolomics is used to study the relationship between metabolism and changes in immunity (O'Neill *et al*, 2016; Ayres, 2020; Troha & Ayres, 2020). For example, Qing *et al* (2020) found that interleukin 6 could modulate glucose metabolism and regulate immunometabolic reprogramming under conditions of acute stress. Other studies have also shown a critical link between metabolic regulation and cytokine release (Tannahill *et al*, 2013; Bambouskova *et al*, 2018; Mills *et al*, 2018). Additionally, metabolic pathways discovered through metabolomics have been found to modulate host responses to different viral infections (Gulati *et al*, 2015; Chan *et al*, 2019; Song *et al*, 2020).

Due to the complexity and diversity of the immune system's regulations, as well as the advancement of systems biology, a single omics study is often insufficient to fully understand it (Eckhardt *et al*, 2020). As shown in Fig 4A, metabolomics, the combination of metabolomics with genomics, transcriptomics (single-cell), and proteomics, allows for the exploration of biological mechanisms from multiple perspectives. Systems immunology, which enables the complementary validation of multiomics data, can reveal the relationship between immunological molecular regulation and phenotypes, as well as identify important metabolic pathways, genes, proteins, and metabolic markers for further experimental analysis (Burel *et al*, 2016; Bakker *et al*, 2018; Koeken *et al*, 2021). A search of PubMed using the keywords "immunity" and "metabolomics" from 2003 to 2021 yielded over 4,300 peer-reviewed publications. As shown in Fig 4B, it is clear that metabolomics has been widely used in immunological research in recent years, with over 1,000 publications in the year 2021 alone. Additionally, it has been found that metabolomics combined with other systems biology methods has also been increasingly applied in immunological studies. The highest number of publications combining metabolomics and genomics in immunity was over 250 articles published in 2021.

In immunological studies, metabolites obtained through metabolomics analysis are often associated with immune phenotypes. To identify the relationship between these two factors, researchers first map the immune phenotypes and all metabolites to each other. Then, they use correlation coefficient methods, such as calculating *Pearson's* or *Spearman's rank* correlation coefficient to determine the correlation between each immune phenotype and each metabolite (one-to-one) or select the significantly different/high abundance metabolites for further. Finally, they use functional analysis, cluster analysis, and network analysis to interpret the data. For example, in previous studies, researchers calculated the association between metabolites and immune phenotypes to identify several metabolites that are significantly correlated, then continued to analyze the correlation between these metabolites and genes, and finally uncovered the causal roles of metabolites in disease (Bakker *et al*, 2018; Chu *et al*, 2021). In another study, Xiao *et al* (2021) first analyzed the correlations between cytokines and metabolites through linear regression models, and further analysis revealed immunometabolic reprogramming in COVID-19. In addition, Abdrabou *et al* (2021)

performed cross-correlation between the transcript abundance of all genes expressed in the samples and infection-associated metabolites to identify possible biological relationships. Furthermore, Nath *et al* (2017) used weighted gene coexpression network analysis (WGCNA) to find the co-expressed gene modules, then enriched by gene ontology (GO) to obtain immunity-related genes, and finally performed correlation analysis with metabolites. Lee *et al* (2022) integrated plasma metabolomics data and transcriptional networks within circulating immune cells identified through single-cell RNA-seq analysis to uncover metabolic changes in COVID-19 patients. In the study of Bakker *et al* (2018), machine learning approaches were used to integrate multiomics data to predict immune functions.

On the contrary, a differential analysis of each omics is performed in a single omics study. This includes statistical analysis of metabolites or genes that are different between groups. Association analysis and pathway analysis are conducted on these differential features. The advantage of this type of analysis is that it allows for the detection of the same enzyme-encoding genes in both the metabolome and transcriptome, which increases the reliability of the features. Additionally, the different genes detected by each omics complement each other between the two omics. Examples of this include proteomics/transcriptomics and metabolomic studies on COVID-19 (Shen *et al*, 2020; Bi *et al*, 2022; Li *et al*, 2022) and the integration of metabolomics and microbiomics to identify critical

Table 1. Major computational tools available for multiomics data integration (sorted alphabetically by tools' name).

Methods/ Tools	URL	Reference	Brief descriptions
BioCyc	https://biocyc.org/	Caspi *et al* (2016)	MetaCyc is a database of metabolic pathways and enzymes (proteins/genes).
CoExp	https://rytenlab.com/coexp	Garcia-Ruiz *et al* (2021)	CoExp is a web server for the exploitation of coexpression networks.
iOmicsPass	https://github.com/cssblab/iOmicsPASS	Koh *et al* (2019)	iOmicsPASS is a tool for network-based multiomics data integration for predictive subnetwork discovery.
MAINE	http://maine.ibemag.pl/#exemplaries	Gruca *et al* (2021)	MAINE is a web server for multiomics feature selection and data exploration.
Mergeomics	http://mergeomics.research.idre.ucla.edu/	Ding *et al* (2021a)	The Mergeomics web server is a flexible online tool for integrating multiomics data to clarify disease networks and predict therapeutics.
MetExplore	https://metexplore.toulouse.inrae.fr/metexplore2/	Cottret *et al* (2018)	MetExplore is a web server for metabolic network curation, network exploration, and omics data analysis.
MetScape	http://metscape.ncibi.org/	Gao *et al* (2010)	MetScape is a tool for metabolites, genes, and pathways that integrates KEGG data.
MiBiOmics	https://shiny-bird.univ-nantes.fr/app/Mibiomics	Zoppi *et al* (2021)	MiBiOmics is a web-based tool that implements classical ordination techniques and the inference of omics-based (multilayer) networks.
mixKernel	http://mixomics.org/mixkernel/	Mariette and Villa-Vialaneix (2018)	mixKernel is a tool that integrates multiple datasets of various types into a single exploratory analysis.
mixOmics	http://mixomics.org/	Rohart *et al* (2017)	mixOmics is a tool for omics feature selection and multiple data integration.
MOFA	https://github.com/bioFAM/MOFA	Argelaguet *et al* (2018)	MOFA is a tool for unsupervised integration of multiomics datasets.
MONGKIE	http://yjjang.github.io/mongkie/	Jang *et al* (2016)	MONGKIE is an integrated tool for network analysis for multiomics data.
MoSBi	https://github.com/tdrose/mosbi	Rose *et al* (2022)	MoSBi is a tool that could automate signature mining for molecular stratification and subtyping.
MultiSLIDE	https://github.com/soumitag/multiSLIDE	Ghosh *et al* (2021)	The multiSLIDE web server allows users to explore related elements of biological pathways in multiomics data.
NeDRex	https://api.nedrex.net/	Sadegh *et al* (2021)	NeDRex is a platform for integrating and interacting with data from different sources, including information on genes, drugs, therapeutic targets, diseases, and their relationships.
OmicsNet	https://www.omicsnet.ca/	Zhou *et al* (2022)	OmicsNet a tool for multiomics integration and network visual analytics.
PaintOmics	https://paintomics.org/	Liu *et al* (2022b)	PaintOmics web server allows for the integrative study of multiomics datasets, which are backed by numerous pathway databases.
pwOmics	https://bioconductor.org/packages/pwOmics/	Wachter and Beissbarth (2015)	pwOmics is an R package for pathway-based integration of time-series omics data using public database knowledge.
SUMMER	https://bitbucket.org/salkigc/summer	Huang *et al* (2020)	SUMMER is a tool that has key metabolic reactions and relevant underlying biological pathways.
WGCNA	https://cran.r-project.org/web/packages/WGCNA/index.html	Langfelder and Horvath (2008)	WGCNA is an R package for weighted correlation network analysis.

features conduct association and functional analysis (Raijmakers *et al*, 2020). Wozniak *et al* (2020) also used binary comparisons and clustering and network-based approaches to identify disease associations within sample groups, providing a comprehensive view of the early host response to *Staphylococcus aureus* bacteremia.

Computational tools for multiomics data integration

In recent years, advances in systems immunology technologies have resulted in a large number of complex datasets. It is crucial to extract biologically meaningful features from these datasets in order to solve immunological problems or implement precision medicine. Therefore, we reviewed the computational methods or tools commonly used to integrate metabolomics with other multiomics data (as shown in Table 1). These methods/tools will enhance a comprehensive understanding of the metabolic processes and immunity of disease.

Analytical methods that integrate metabolomics and other omics can be divided into three strategies based on prior knowledge, network methods/clustering, and data-based integration. The strategy based on prior knowledge uses knowledge from databases or scientific literature to establish connections on various omics data and construct regulatory networks. These tools include the R package pwOmics (Wachter & Beissbarth, 2015), the BioCyc database (Caspi *et al*, 2016), the MetExplore web server (Cottret *et al*, 2018), and software like MetScape (Gao *et al*, 2010) and MONGKIE (Jang *et al*, 2016). Moreover, the strategy based on network methods/clustering is used to study the association between co-expressed network modules, and combined with prior knowledge, to further explore the relationship between network modules and diseases. These analytical tools for this strategy include the WGCNA R software package (Langfelder & Horvath, 2008) and web servers like iOmicsPass (Koh *et al*, 2019), OmicsNet (Zhou *et al*, 2022), MiBiOmics (Zoppi *et al*, 2021), PaintOmics (Liu *et al*, 2022b), NeDRex (Sadegh *et al*, 2021), and CoExp (Garcia-Ruiz *et al*, 2021). The last is the data-based integration strategy, which relies on the characteristics of the data itself, and uses machine learning models or statistical models to find the relationship between the omics data. R packages for this strategy include MOFA (Argelaguet *et al*, 2018), mixOmics (Rohart *et al*, 2017), mixKernel (Mariette & Villa-Vialaneix, 2018), and MoSBi (Rose *et al*, 2022). Web servers for this strategy include MAINE (Gruca *et al*, 2021), MultiSLIDE (Ghosh *et al*, 2021), Mergeomics (Ding *et al*, 2021a), and SUMMER (Huang *et al*, 2020).

New developments and discussion

The metabolic pathways in immune cells are closely related to specific immune functions and cellular states in health or disease (Buck *et al*, 2017; Geltink *et al*, 2018). Traditional methods for detecting a large number of cells may not accurately reflect the situation of a single cell due to the heterogeneity of cells and rapid turnover of metabolites. Therefore, it is increasingly necessary to detect metabolites in a single cell (Evers *et al*, 2019). Single-cell metabolomics can directly obtain the metabolite information of a single cell and provide insight into the relationship between the physiological process of a single cell and its chemical composition (Kumar *et al*, 2020; Shrestha, 2020). In recent years, mass spectrometry imaging (MSI) and spatial resolution MS analysis have made significant progress in achieving spatial metabolomics at the single-cell scale (Petras *et al*, 2017; Alexandrov, 2020). Hartmann and Bendall (2020) used their established MIBI-TOF platform to combine single-cell metabolic profiling, immune cell phenotype, functional status, cell–cell interactions, and location within tissues. Wang *et al* (2022) developed an advanced spatially high-resolution metabolomics approach capable of achieving single-cell-level resolution *in situ* to interpret cell-type-specific metabolic dynamics in the context of the structure and metabolism of neighboring cells. Rappez *et al* (2021) developed an open-source method for *in situ* single-cell metabolomics using matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry, combined with fluorescent signals and morphospatial features, to perform high-throughput, *in situ* metabolome analysis at the single-cell level. In addition, Artyomov and Van den Bossche (2020) provide a detailed review of single-cell application techniques and their general principles for studying immune metabolism, outline the metabolic heterogeneity of immune cells, and discuss limitations of current immune techniques and future directions for exploration. The rapidly growing field of single-cell metabolomics will help to understand the metabolic pathways of immune cells and the mechanisms that lead to immune dysfunction and disease development when metabolism is abnormal.

The metabolic changes that occur in immune cells are dynamic (Loftus & Finlay, 2016). A theoretical model explaining the dynamics of this process will allow us to understand the consequences of changes in the levels of enzymes, metabolites, or regulators in this key cellular process. However, creating such a model is challenging due to the thousands of metabolic reactions that occur within cells (Medina, 2020). Purohit *et al* (2022) provide a thorough overview of current metabolic modeling methods used to study cellular

Box 1. In need of answers

i Can metabolomics research and our current understanding of metabolic processes in immune regulation provide potential therapeutic targets or interventional strategies for the treatment of infectious diseases?

ii A variety of novel statistical analysis algorithms and methods for metabolomics data processing are now available, but different methods would produce different results. Therefore, how should we choose the most appropriate methods to obtain robust and accurate results?

iii Systems immunology can reveal the relationship between immunological molecular regulation and phenotype from multilevel omics data. However, integrating multiomics data can be computationally intensive and challenging. Is it possible to develop a convenient and reliable integration pipeline to assist researchers in analyzing multiomics data?

iv Single-cell metabolomics and spatial metabolomics are important in immunology research, but the analysis process can be complex. Can we develop a convenient analytical tool for single-cell metabolomics to promote the wider use of this technology?

v The metabolic changes that occur in immune cells are dynamic, but this process is currently not explained by any theoretical mathematical model. Could Such a model help us to understand the dynamic changes of genes, enzymes, and metabolites in this process and enhance our understanding of immune cell metabolism?

metabolism, summarize the applications and limitations of metabolic models for studying immune metabolism, and introduce mathematical modeling tools that are expected to advance the field. Recent technological advances, such as imaging techniques and multiomics, have provided unprecedented detail on the functioning of the immune system. However, techniques for broader coverage (more metabolites) and improved quantification are still needed in metabolomics. Additionally, due to the complexity of the immune system and the large experimental datasets, it is a great challenge to develop methods to efficiently and quickly estimate parameters in large-scale mathematical models. Further progress in metabolomics and theoretical science will help to advance our understanding of complex immune metabolism (see also Box 1). Effective integration of metabolomics with systems immunology can help to better understand the immune system and may contribute to personalized medicine.

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The authors declare that they have no conflict of interest.

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